Technical method

In situ cytoenzymatic identification of human bone marrow colonies grown in agar: a simple method with automated cytochemistry reagents

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Human bone marrow culture in agar is a widely used technique for the study of haemopoietic progenitor cells in both normal and pathological states.¹ The colonies formed are heterogeneous, consisting of neutrophil, monocyte, eosinophil, and mixed neutrophil monocyte colonies. Methods for differentiating colony types have been described,¹⁻⁸ but they are cumbersome, require removal of the agar from the plate and subsequent desiccation, or the micro-manipulation of individual colonies. Few investigators have therefore reported differential colony counts which may be highly relevant in pathological situations.⁴ Enzyme cytochemistry facilitates the identification of leucocyte types⁹ but the standard techniques require effective cell washing which is extremely difficult in the presence of agar gel. We have therefore adapted cytoenzymatic methods as used in the "Hemalog D" automated differential counter¹⁰ to the staining of agar plates. In the continuous flow methods in the Hemalog D, reagents

are added sequentially; cells do not require washing and there is no extracellular precipitate.

We show that these Hemalog D methods can be modified to perform sequential enzymatic stains on agar plates, making it possible to identify specifically by low power microscopy all of the colony types of the undisturbed agar culture plates.

Methods

Human myeloid colonies are grown in a double layer agar gel system in 30 mm Petri dishes using a modification of the method of Pike and Robinson.¹¹ The 1.0 ml underlayer consists of α -medium, 25% fetal calf serum, and 15% human placental conditioned medium¹² as a source of colony stimulating activity, in 0.5% agar. The 0.5 ml overlayer, containing 10⁵ bone marrow nucleated cells, consists of α -medium and 25% fetal calf serum in 0.3% agar. Culture plates are incubated in 5% CO₂ and analysed routinely at 14 days with the dissecting microscope.

All staining solutions are prepared shortly before use. The ingredients are all Hemalog D reagents (Technicon Instruments Co Ltd, Hamilton Close, Basingstoke, Hants, England). Precise measurement of reagent volumes is unnecessary and disposable syringes are ideal.

Esterase stain for the identification of monocytes

The following reagents (Table) are mixed in the order stated, pausing briefly between each addition. Monocyte dye 2 volumes; monocyte nitrite 2 volumes; monocyte substrate 1 volume; monocyte buffer 5 volumes. This solution is then diluted

"Hemalog D" reagent constituents

Reagent	Technicon reagent No	Effective constituents	g/l
Monocyte dye	T01-0529-38	Sulphuric acid	41.2
		Pararosaniline hydrochloride	10.0
Monocyte nitrite	T01-0530-38	Sodium nitrite	10.0
Monocyte substrate	T01-0680-38	α -naphthol butyrate	3.0
		Saponin	2.0
		Methanol 20% vol/vol	—
Monocyte buffer	T01-0678-38	Sodium cacodylate	70.0
Peroxidase dye	T01-0613-38	2,2'-oxydiethanol 4 chloro-1 naphthol	8.0
Peroxidase substrate	T21-0626	Acetic acid	10.6
	T21-0625	Hydrogen peroxide (10 vol) 3% vol/vol	—
Peroxidase acid	T01-0627-38	Acetic acid	68.6

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eightfold with normal saline. The final solution is gently flooded on to the agar surface and then injected beneath the agar gel using a syringe and blunt fine gauge needle. The culture plate is incubated for 30 min at 37°C and the staining solution is then removed with the syringe and needle.

Peroxidase stain for the identification of granulocytes

The following reagents are mixed in the order stated, pausing between each addition. Peroxidase dye 6 volumes; peroxidase substrate 4 volumes; peroxidase acid 1 volume. This solution is diluted with an equal volume of normal saline and applied to the agar as described above. Incubation is for 15 min at 37°C. The acid staining solution causes opacification of the agar matrix and this is reversed by removal of the staining solution and the addition of 0.25 M sodium hydroxide.

Peroxidase stain with resorcinol inhibition for the differentiation of neutrophil and eosinophil colonies

The peroxidase stain is performed as above except that resorcinol has already been added to the peroxidase dye to a concentration of 1.5 g/l. Resorcinol at this concentration specifically inhibits eosinophil peroxidase.¹³

Combined esterase and peroxidase stain

Esterase staining is carried out as described above, but after removal of the staining solution from the plate, the agar is gently irrigated with normal saline to remove excess staining solution. The peroxidase stain is then performed as described above.

Results and discussion

The esterase stain displays monocyte colonies as

disperse colonies which contain large red-brown cells (Fig. 1). Granulocytic colonies may occasionally have weak esterase activity, but this is insufficient to lead to confusion particularly where indirect illumination is used to examine the culture plates.

The peroxidase method stains granulocyte colonies grey/black (Fig. 2). At the low pH used (pH 3.4) eosinophil colonies are stained more heavily than neutrophil colonies and monocyte colonies have extremely weak peroxidase activity which is not seen on indirect illumination (Fig. 3). To facilitate the differentiation of neutrophil and eosinophil colonies resorcinol may be added to the peroxidase dye. This specifically inhibits eosinophil peroxidase causing eosinophil colonies to appear as compact unstained cell aggregates with a yellowish tinge (Fig. 4). The eosinophilic nature of these unstained colonies was verified by removing these colonies from the agar and performing May-Grunwald-Giemsa staining on dried squash preparations.

The combined esterase and peroxidase stains may be used to identify granulocytic and monocytic colonies in the same culture dish (Fig. 5). The occasional mixed neutrophil-monocyte colony can also be readily identified (Fig. 6). T-cell colonies which may be present when phytohaemagglutinin-leucocyte conditioned medium is used as a source of colony stimulating activity¹⁴ do not exhibit esterase or peroxidase activity and may be readily shown by this combined method. A combined esterase and resorcinol-peroxidase method may be used, but the results are variable and it is our practice to use the resorcinol-peroxidase stain alone on a replicate culture plate.

With these methods it is now possible to perform differential colony counts routinely on all bone marrow culture plates.

Fig. 1 Esterase stain using indirect illumination. Two unstained granulocytic colonies are seen on the left. On the right is a monocyte colony consisting of dispersed large esterase positive cells.

Fig. 2 Peroxidase stain before clarification with sodium hydroxide. Grey/black peroxidase positive colonies are visible to the naked eye.

Fig. 3 Peroxidase stain using indirect illumination. On the left is an unstained monocyte colony and to the right of this, three granulocytic colonies. The colony on the extreme right, staining most intensely, is probably an eosinophil colony. The other two granulocytic colonies probably consist of neutrophils.

Fig. 4 Peroxidase stain with resorcinol using indirect illumination. On the left is a peroxidase positive neutrophil colony. In the centre is a disperse unstained monocyte colony, and on the right is a compact negatively stained eosinophil colony with a yellow hue.

Fig. 5 Combined esterase and peroxidase stain using direct illumination. On the left is a granulocytic colony, and on the right two monocytic colonies.

Fig. 6 Combined esterase and peroxidase stain using direct illumination. A mixed neutrophil-monocyte colony is seen above a large granulocytic colony.

Figs. 1, 3, 4, 5, and 6 are $\times 40$ photomicrographs taken without removing the agar from the culture plates.

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Letters to the Editor

Whole blood filtration

Since the article "Effect of total leucocyte count on whole blood filterability in patients with peripheral vascular disease," by MJ Alderman, Anne Ridge, AA Morley, RG Ryall and JA Walsh¹ refers to both our method of red cell filtration (RCF) and our findings in peripheral vascular disease, we would like to comment.

The original method,² developed in our laboratory in 1975, has a number of drawbacks. In addition to the influence of white blood cells (WBC) correctly observed by the authors of the recent article in your journal, it submits the red cells to unphysiologically high shear stresses and also can be affected by red cell aggregation. For this and other reasons, we have modified our technique in 1979,³ the three most important changes being: (i) the absence of external pressure; (ii) the reduction of WBC and platelets in the sample to be filtered; and (iii) the use of a 5% red cell suspension for filtration. Under these conditions using lithium heparin as anticoagulant, we find the reproducibility increased and the assessment of the in vivo deformability is probably better. The influence of WBC can still be demonstrated by this method if the white cells are added artificially to the

sample (unpublished data). However, the preparation of the sample includes the elimination of at least 75% of the WBC (unpublished data). In this context, it is noteworthy that the authors had to use WBC concentrations outside the physiological range in order to demonstrate the influence of WBC on RCF. In pathologically high concentrations, WBC represent a potential blocker not only for the in vitro test, but also for the microcirculation in vivo.⁴

The finding that RCF is reduced in peripheral vascular disease has been reported by us⁵ and others.⁶ When eliminating the effect of WBC, the RCF of claudicants is still impaired⁶ (and own unpublished data). Hence, we cannot confirm the authors' observation that the decrease of RCF is abolished when correcting for a standard WBC count.

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Dr MJ Alderman and his colleagues reply as follows:

In reply to Drs Dormandy and Ernst we believe that the original technique of whole filtration, introduced by Dr Dormandy's group, was potentially a very attractive means of estimating a physio-